Analysis of in vitro and in vivo products of the TMV 30kDa open reading frame using antisera raised against a synthetic peptide

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The peptide Tyr-Ser-Glu-Ala-Thr-Val-Ala-Glu-Ser-Asp-Ser-Phe (the predicted C-terminal 11 amino acids of the TMV 30kDa open reading frame plus an additional N-terminal Tyr residue) was synthesized by solid phase methods and used to raise antisera in rabbits. These antisera precipitated 4 major proteins (p30, p28, p19 and 18.5kDa) from in vitro translation products of TMV short rod RNA, but only one, of apparent M_r = 30500, from TMV-infected tobacco protoplasts. This protein was made between 8 and 16 h post infection, and had [^{35}S]Met-labelled tryptic peptides identical to those of in vitro synthesized p30.

Antipeptide antiserum *Solid phase peptide synthesis* *Tobacco mosaic virus* *In vitro translation* *Tobacco protoplast* *Local lesion spreading*

1. INTRODUCTION

The coding capacity of many plant viruses with messenger sense RNA genomes has been studied by using viral RNA to direct protein synthesis in vitro. Major translation products often correspond to predicted open reading frames (ORFs) where the RNA sequence is known, but detection of non-structural proteins to prove their identity with in vitro translation products has proved difficult. An attractive new approach to this problem, given the RNA sequence, is to use synthetic peptides predicted to occur in the amino acid sequence to raise antisera capable of precipitating the native protein [1]. We have used this method to produce antisera to protein p30 of TMV: the predicted product of an open reading frame (ORF) encoded by nucleotide residues 4903–5706 on TMV RNA (76.7–89.2% of the genome from the 5′-end) [2]. Genetic experiments implicate a product of this ORF in the spreading of necrotic local lesions [3,4], but at least 3, and possibly as many as 6 overlapping protein products can be found in vitro [5]. A protein of M_r = 30000–31000 has been reported in TMV infected protoplasts [6] and leaves [7], but its ephemeral nature has prevented further characterisation, and leaves open the existence of other products. We expected that antisera to p30 peptides would be useful as probes to identify and enumerate p30-related proteins in vivo, and for other studies.

2. MATERIALS AND METHODS

2.1. *Solid phase peptide synthesis*

The desired peptide was synthesized on a cross-linked polydimethylacrylamide resin (1 g) functionalized with internal reference *t*-butoxycarbonyl (Boc)-norleucine (0.12 meq/g) (UCB Bioproducts, Brussels) as follows [8,9]:

(i) cleavage of the Boc-groups using N-HCl/AcOH and neutralisation with 10% diisopropylethylamine in freshly distilled DMF;

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(ii) acylation with the reversible linkage agent, *p*-hydroxymethylphenoxyacetic acid trichlorophenyl ester in the presence of hydroxybenzotriazole (1 eq.) (70 min);
(iii) esterification with freshly prepared fluorenylmethoxycarbonyl (Fmoc)-Phe anhydride in the presence of *N*-methylmorpholine (1 eq.) and 4-dimethylaminopyridine [10] (30 min);
(iv) a cycle of deprotection with 20% piperidine in DMF (3 + 7 min); followed by acylation with a 6-fold excess of the appropriately protected Fmoc-amino acid derivatives in DMF (60 min).

An aliquot (0.104 g) of the dried resin was cleaved with 95% aq. CF_3CO_2H for 1.5 h and the product (18.6 μmol, 98% cleavage) chromatographed on DEAE-cellulose (DE52) using a linear gradient of 0.01–0.3 M aq. NH_4HCO_3, pH 8.1. The major peak was collected, freeze dried (yield = 13.82 μmol = 74%) and characterized by high performance liquid chromatography (HPLC) and amino acid analysis (fig.1).

2.2. *Coupling and immunisation*

o-Toluidine (3.4 mg) was bis-diazotised as described for benzimide [11] and used to couple the peptide (6 mg) to carrier bovine serum albumin (15 mg) [1]. New Zealand White rabbits were immunized by intramuscular or multiple subcutaneous injections of one-eighth aliquots of conjugate (0.5 ml) in Freund's complete adjuvant (1 ml) and up to two intramuscular booster injections of half these amounts at various intervals up to 4 months.

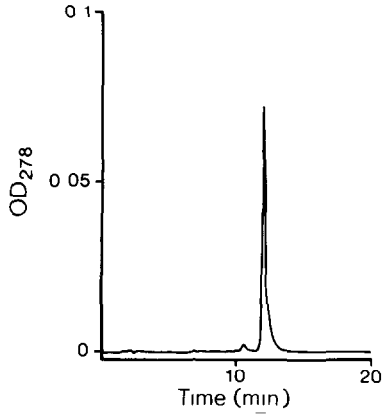

Fig.1. Analytical reversed-phase HPLC was carried out on μBondapak C_{18} using a linear gradient of 10–25% B over 20 min when pump A was delivering 0.01 M NH_4OAc (pH 4.5) and pump B 90% acetonitrile/10% A, with a flow rate of 1.5 ml/min. The profile at 230 nm was similar to that shown at 278 nm. Amino acid analysis of the DE 52 purified product showed: Phe 0.90, Ser 2.49, Asp 1.00, Glu 2.09, Ala 2.00, Val 0.99, Thr 0.86 and Tyr 0.94.

2.3. *In vitro translation*

A crude TMV short rod preparation was made by 'differential resuspension' and CsCl-banding essentially as in [12]. Electron microscopy of the particles showed 58% less than half length (probably mostly broken rods) and 26% full length (total scored, 159). RNA was extracted, translated [13] and products fingerprinted as in [5].

2.4. *Protoplast procedures and immunoprecipitation*

Protoplasts isolated from *N. tabacum* cv Xanthi nc were inoculated with TMV RNA using the PEG-Ca^{2+} method [14] and incubated at 5 × 10^5/ml in medium modified from [15] containing 0.3–1.0 mCi/ml [^{35}S]Met (>1000 Ci/mmol, Amersham). Detailed procedures will be described elsewhere (in preparation). Detergent lysis and immunoprecipitation with carrier Pansorbin (Calbiochem) used published procedures [16,17] indicated in the text and figure legends.

3. RESULTS

We previously showed that the 30kDa ORF of TMV is translated in vitro into at least 3 overlapping proteins with staggered N termini and probably identical C termini [5], so a synthetic peptide corresponding to the C terminus of the 30kDa ORF (which is hydrophilic and so may be exposed in the native protein) should elicit antibodies capable of precipitating all 3 proteins, and any other C coterminal products. Peptides corresponding to protein termini have been used previously to raise antisera to native proteins [1]. Thus, the sequence chosen for synthesis was the C terminal undecapeptide of p30: Ser-Glu-Ala-Thr-Val-Ala-Glu-Ser-Asp-Ser-Phe [2], which was synthesized with an extra Tyr residue at the N terminus, coupled to BSA via this tyrosine using bis-diazotised *o*-toluidine, and used to raise antisera as described in section 2.

3.1. *Immunoprecipitable products of the 30kDa ORF in vitro*

Antisera prepared from four rabbits immunised by different protocols all precipitated 4 major proteins from the reticulocyte lysate translation products of RNA isolated from a short rod-enriched virus preparation (fig.2). These comigrated with in vitro products whose synthesis was resistant to m^7GTP, a characteristic of products of the 30kDa ORF [5,7,18]. The 3 largest of these proteins (known as p30, p28 and p19 from their molecular masses) have been shown by peptide mapping to be overlapping translation products of the 30kDa ORF [5]; this experiment confirms their derivation and relationship to the fourth protein (18.5 kDa) and proves that all 4 proteins are C coterminal, as was previously inferred. Control experiments showed this precipitation to be specific since it was blocked by preincubation of antisera with excess peptide (qv fig.3), so distinguishing it from a variable background precipitation of additional proteins by immune antisera, or of the p30 family by preimmune or irrelevant (such as antiferritin) antisera. Backgrounds were reduced using conditions as in [17] rather than those in [16], but yields were also reduced.

3.2. *Characterisation of a product of the 30kDa ORF in vivo*

In order to screen for the translation products of

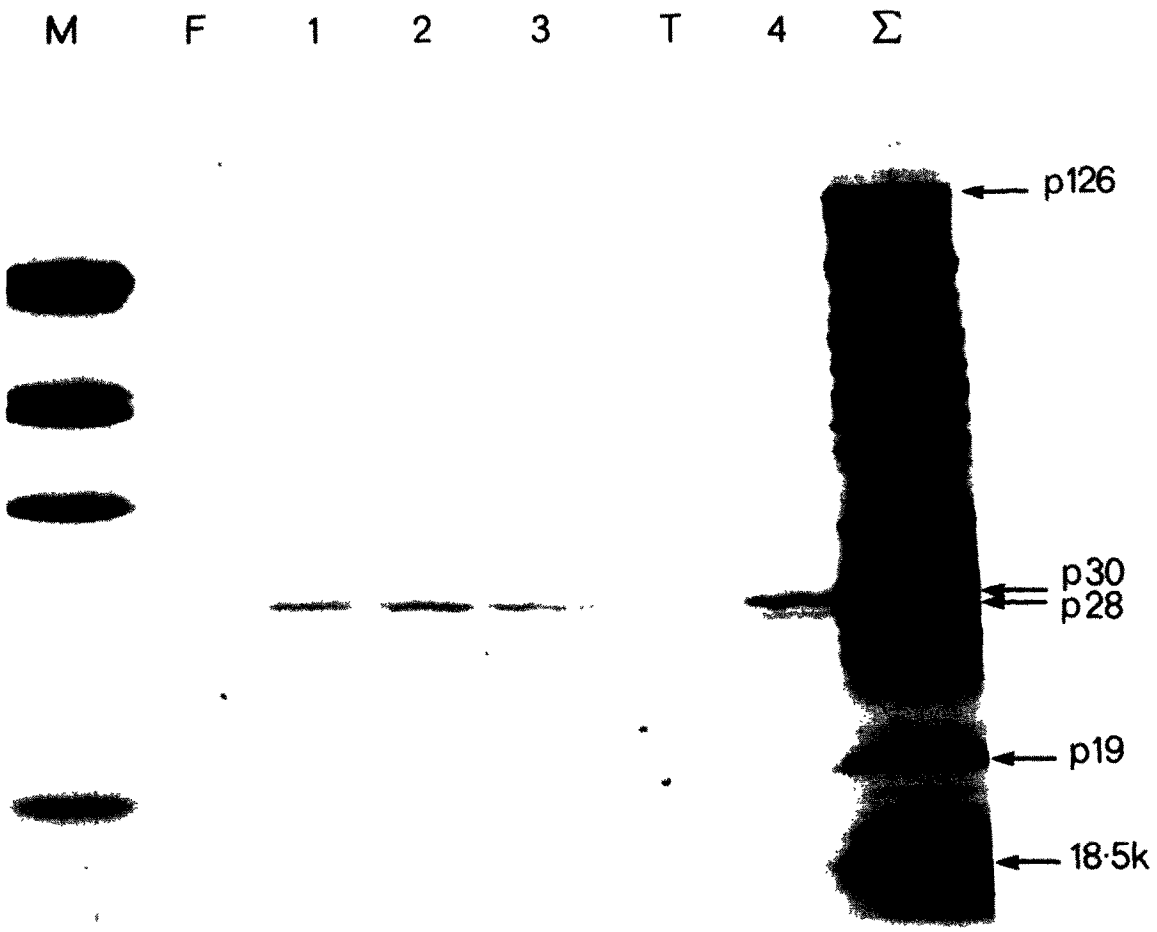

Fig.2. In vitro products of TMV 'short rod' RNA translation in the messenger dependent reticulocyte lysate, immunoprecipitated as in [17]. Σ, starting material; M, markers (88kDa, 56kDa (doublet), 40kDa and 21kDa); F, antiferritin antiserum; T, TMV antiserum; 1,2,3,4, antipeptide antisera from different rabbits.

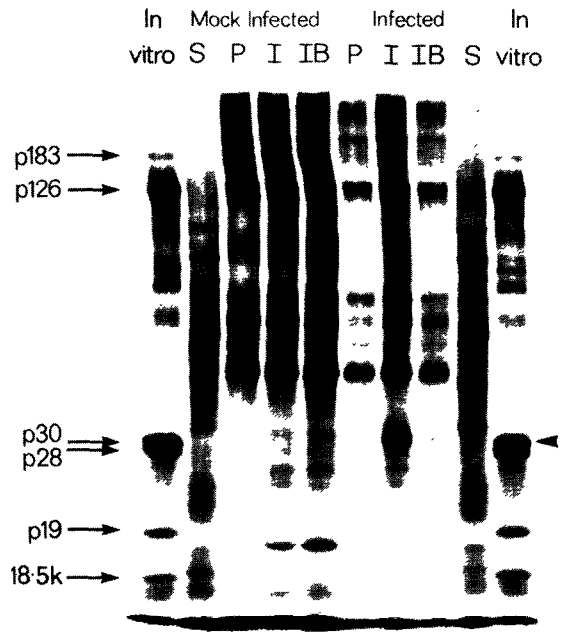

Fig.3. Detergent lysates of tobacco protoplasts infected with TMV RNA, or mock infected, 28 h previously were immunoprecipitated as in [16]. S, starting material; P, preimmune serum; I, immune serum (R90); IB, I, preincubated with excess peptide (3 $\mu g/\mu l$ of serum). In vitro, reticulocyte translation of 'short rod' RNA in the presence of 1 mM m^7GTP (major products arrowed on the left). Arrowhead on right denotes a specifically immunoprecipitated protein of M_r ~30000 in infected cells.

the 30kDa ORF in vivo, immunoprecipitates were prepared from detergent lysates of tobacco protoplasts infected 28 h previously with TMV RNA, and from mock infected controls. Preliminary experiments revealed limited amounts of p30, so we used the protocol in [16] and prepared 10^7–10^8 cpm of ^{35}S-labelled trichloroacetic acid precipitable protoplast sample for each experiment, distinguishing the background of non-specific material from specific immunoprecipitation with the appropriate controls using preimmune serum and immune serum preincubated with the synthetic peptide. Fig.3 shows that a protein of about M_r = 30500 appeared in immunoprecipitates made using immune serum and a lysate of infected cells, but not in lysates of mock infected cells or with preimmune or peptide blocked sera. It seemed likely that this protein was an in vivo product of the 30kDa open reading frame, although fig.3 shows that it did not exactly comigrate with the in vitro synthesized marker p30. A preliminary experiment showed that mixing in vitro synthesized p30 with protoplasts followed by lysis and immunoprecipitation did not alter its mobility to the in vivo position; nevertheless tryptic peptide mapping (fig.4) showed conclusively that the [^{35}S]methionine-labelled maps of in vitro and in vivo synthesized p30 were indistinguishable.

Twenty-eight hours is late in TMV infection, so to examine the possibility that other p30-related proteins were made at other times, samples of mock-infected and infected protoplasts were immunoprecipitated at 8, 16, 24 and 32 h post infection. The results (fig.5) showed that the amount of p30 present increased sharply between 8 h (when it was barely detectable) and 16 h, and that little additional accumulation occurred after that. No other p30-related proteins were detectable at any time. This result suggests that p30 synthesis begins close to the onset of exponential growth at about 8 hpi [15], but that there is little or no nett synthesis of p30 late in infection, unlike coat protein. More detailed experiments would be needed to determine the kinetics of p30 synthesis relative to those of the other TMV proteins.

4. DISCUSSION

These results show that antisera to TMV protein p30 can be successfully raised by the synthetic peptide strategy. Solid phase methods have now advanced to the point where almost any desired peptide can be synthesized routinely. Therefore this method may prove very useful in the identification and analysis of plant viral proteins, where the gene sequence is often more accessible than the gene product.

Our analysis of products of in vitro translation using the anti-p30 antisera confirms previous work showing a family of overlapping proteins [5]. Whereas enumeration of these proteins previously depended on exhaustive peptide mapping, the antisera precipitate all these related proteins in a single experiment, and prove that they are C coterminal.

In contrast, similar experiments reveal only a single product in vivo. This protein appears to

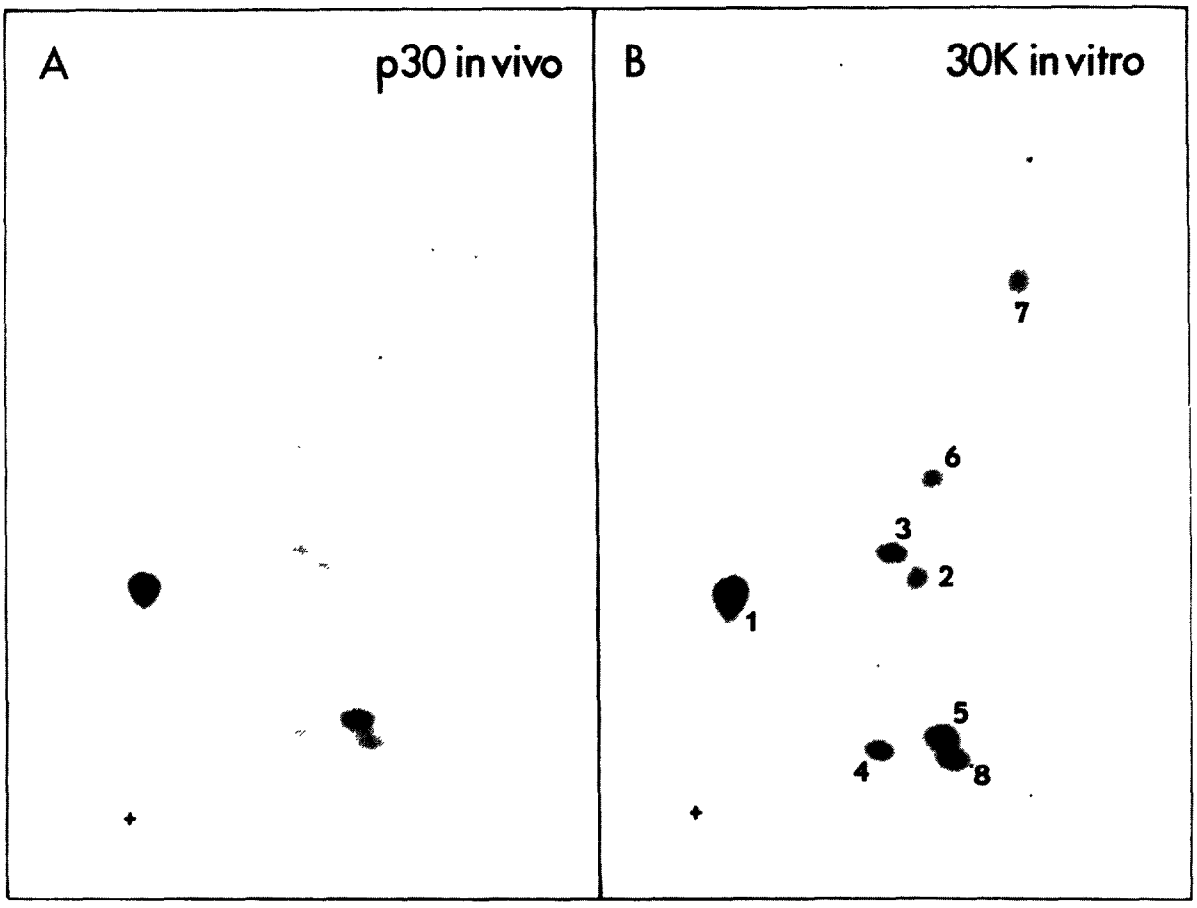

Fig.4. Tryptic peptide maps of [^{35}S]Met-labelled p30 immunoprecipitated from infected tobacco protoplasts (A) compared with that of the 30 kDa product of in vitro translation of 'short rod' RNA (B). Spot numbering as in [5].

migrate slightly slower on gels than in vitro synthesized p30, but tryptic peptide mapping (fig.4) shows no detectable differences in the Met-containing peptides. The mobility difference could be due to a post-translational modification, but if so the modification is probably not within a Met-containing tryptic peptide. No smaller p30-related proteins were detected in any of our protoplast experiments, although p30 itself was expressed at a moderately low level (in the range of 0.1–0.01% of the total trichloroacetic acid-precipitable ^{35}S incorporation), and we estimate that our experiments would be insufficiently sensitive to detect expression of other products at below a few percent of this level. The discrepancy between the in vitro and in vivo results may be due to template breakdown in vitro, but if so the previously documented similarities between p30 synthesis in vitro and that of the smaller proteins [5,18] suggest that the in vitro expression of p30 itself may not properly reflect the in vivo mechanism. Alternatively, the short rod RNAs which direct synthesis of the smaller p30-related proteins may be generated in vivo by errors in subgenomic RNA synthesis, but their protein products may be non-functional and rapidly degraded.

We recently learned that other workers using similar methods have obtained broadly similar results. Their work is being reported elsewhere [19].

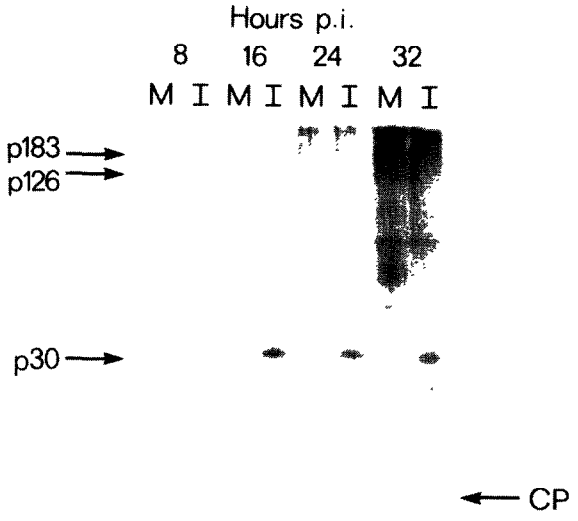

Fig.5. Time course of [^{35}S]Met-labelled p30 synthesis in TMV-infected tobacco protoplasts. M, mock infected; I, infected. Positions of in vitro synthesized markers on the left, that of coat protein synthesized in an aliquot of the same protoplasts labelled with [^{3}H]Leu on the right. Labelling was continuous; total trichloroacetic acid-precipitate incorporation increased linearly for 16 h and at about 50% of the initial rate for the next 16 h.

REFERENCES

[1] Walter, G., Scheidtmann, K.-H., Carbone, A., Laudano, A.P. and Doolittle, R.F. (1980) Proc. Natl. Acad. Sci. USA 77, 5197–5200.
[2] Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J. and Karn, J. (1982) Proc. Natl. Acad. Sci. USA 79, 5818–5822.
[3] Leonard, D. and Zaitlin, M. (1982) Virology 117, 416–424.
[4] Zimmern, D. and Hunter, T. (1983) EMBO Journal, in press.
[5] Hunter, T., Jackson, R. and Zimmern, D. (1983) Nucleic Acids Res. 11, 801–821.
[6] Beier, H., Mundry, K.W. and Issinger, O.-G. (1980) Intervirology 14, 292–299.
[7] Joshi, S., Pleij, C.W.A., Haenni, A.-L., Chapeville, F. and Bosch, L. (1983) Virology 127, 100–111.
[8] Arshady, R., Atherton, E., Clive, D.L.J. and Sheppard, R.C. (1981) J. Chem. Soc. Perkin 1, 529–537.
[9] Atherton, E., Logan, C.J. and Sheppard, R.C. (1981) J. Chem. Soc. Perkin 1, 538–546.
[10] Atherton, E., Benoiton, N.L., Brown, E., Sheppard, R.C. and Williams, B.J. (1981) J. Chem. Soc. Chem. Comm. 336–337.
[11] Bassiri, R.M. and Utiger, R.D. (1972) Endocrinology 90, 722–727.
[12] Whitfeld, P.R. and Higgins, T.J.V. (1976) Virology 71, 471–485.
[13] Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247–256.
[14] Dawson, J.R.O., Dickerson, P.E., King, J.M., Trim, A.R.H. and Watts, J.W. (1978) Z. Naturforsch. 33C, 548–551.
[15] Aoki, S. and Takebe, I. (1969) Virology 39, 439–448.
[16] Papkoff, J., Lai, M.H.-T., Hunter, T. and Verma, I.M. (1981) Cell 27, 109–119.
[17] Semler, B.L., Hanecak, R., Dorner, A.J., Anderson, C.W. and Wimmer, E. (1983) Virology 126, 624–635.
[18] Pelham, H.R.B. (1979) FEBS Lett. 100, 195–199.
[19] Ooshika, I., Watanabe, Y., Meshi, T., Okada, Y., Igano, K., Inouye, K. and Yoshida, N. (1983) Virology, in press.